MTB Diff Real-TM

Handbook

Real-Time Amplification test for the qualitative detection and differentiation of *M.tuberculosis, M.bovis and M. bovis BCG*

REF B41-50FRT

REF TB41-50FRT

Σ 50

NAME

MTB Diff Real-TM

INTRODUCTION

Tuberculosis (abbreviated as **TB** for *tubercle bacillus*) is a common and deadly infectious disease caused by mycobacteria, mainly *Mycobacterium tuberculosis*. Tuberculosis most commonly attacks the lungs (as pulmonary TB) but can also affect the central nervous system, the lymphatic system, the circulatory system, the genitourinary system, bones, joints and even the skin. Other mycobacteria such as *Mycobacterium bovis*, *Mycobacterium africanum* and *Mycobacterium microti* can also cause tuberculosis. Over one-third of the world's population has been infected by the TB bacterium, and new infections occur at a rate of one per second. Not everyone infected develops the full-blown disease; asymptomatic, latent TB infection is most common. However, one in ten latent infections will progress to active TB disease, which, if left untreated, kills more than half of its victims.

Early diagnosis of tuberculosis makes effective treatment possible and increases the probability of clinical outcome owing to quite effective antituberculosis therapy, however the tuberculosis diagnosis has certain difficulties. According to international standards, tuberculosis diagnosis must be confirmed either by bacteriology or by histology studies, but the bacteriological methods do not always allow to detect Mycobacterium tuberculosis in people affected with pulmonary tuberculosis and especially with extrapulmonary tuberculosis.

The application of molecular biology methods allow to overcome the difficulties in the diagnosis of Mycobacterium tuberculosis, but due to the biological peculiarities of this microorganism and immune response of human organism, tuberculosis can not be diagnosed only by one method.

INTENDED USE

The development of test to differentiate between infection with *Mycobacterium tuberculosis* or *Mycobacterium bovis* and vaccination with *M. bovis* BCG could greatly assist in the diagnosis of early infection as well as enhance the use of tuberculosis vaccines on a wider scale.

PRINCIPLE OF ASSAY

kit **MTB Diff Real-TM** is a Real-Time Amplification test for the qualitative detection and differentiation of *M.tuberculosis, M.bovis and M. bovis BCG* in the sputum, urine, blood, bronchial lavages, tissue and other biological materials. DNA is extracted from samples, amplified using Real Time Amplification and detected using fluorescent reporter dye probes specific for *M. tuberculosis, M.bovis, M. bovis BCG* and IC. *M. tuberculosis* IC is DNA fragment of IS 6110 insertion of *Mycobacterium tuberculosis* modified and cloned in bacteriophage λ, containing DNA fragments used in the kit as matrix for primers. Internal Control (IC) serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition.

M. tuberculosis is detected on the Fam (Green) channel, *M.bovis* and *M. bovis BCG* on the Joe (Yellow)/HEX/ TET/Cy3 channel, *M. bovis BCG* on the Rox (Orange)/TexasRed channel and IC on the Cy5 (Red) channel.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B41-50FRT)

Part N° 2 – **"MTB Diff Real-TM":** Real Time amplification

- **PCR-mix-1 Diff**, 2 x 0,3 ml;
- **PCR Buffer Flu**, 2 x 0,2 ml;
- **TaqF Polymerase**, 0,03 ml;
- **MTB Diff C+**, 0,1 ml;
- **DNA-buffer**, 0,5 ml;
- **Negative Control C-**, 1,2 ml;*
- **MTB IC** , 0,5 ml.**

Contains reagents for 55 tests.

* *must be used in the isolation procedure as Negative Control of Extraction.*

** *add 10 µl of Internal Control during the DNA purification procedure directly to the sample/lysis mixture*

Module No.2: Complete Real Time PCR test with DNA purification kit (TB41-50FRT)

Part N° 1 – **"DNA-Sorb-B":** Sample preparation

- **Lysis Solution**, 15 ml;
- **Washing Solution 1**, 30 ml;
- **Washing Solution 2**, 50 ml;
- **Sorbent**, 1,25 ml;
- **DNA-eluent**, 5 ml.

Contains reagents for 50 extractions

Part N° 2 – **"MTB Diff Real-TM":** Real Time amplification

- **PCR-mix-1 Diff**, 2 x 0,3 ml;
- **PCR Buffer Flu**, 2 x 0,2 ml;
- **TaqF Polymerase**, 0,03 ml;
- **MTB Diff C+**, 0,1 ml;
- **DNA-buffer**, 0,5 ml;
- **Negative Control C-**, 1,2 ml;*
- **MTB IC** , 0,5 ml.**

Contains reagents for 55 tests.

* *must be used in the isolation procedure as Negative Control of Extraction.*

** *add 10 µl of Internal Control during the DNA purification procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (Module No. 1)
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with filters
- 1,5 ml polypropylene sterile tubes
- Biohazard waste container
- Refrigerator, Freezer

Zone 2: Real Time amplification:

- Real Time Thermal cycler
- Reaction tubes
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

Store kit at 2-8°C. **PCR-mix-1** and **TaqF Polymerase** must be stored at -20°C. The kit can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt. Store **DNA-Sorb-B** kit at 2-25°C.

STABILITY

MTB Diff Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

QUALITY CONTROL

In accordance with Sacace’s ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

**** Only for Module No.2***

PRODUCT USE LIMITATIONS

Use of this product should be limited to personnel trained in the techniques of DNA amplification (EN375). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

MTB Diff Real-TM can analyze DNA extracted with **DNA-Sorb-B** (REF K-1-1/B) from:

- *Sputum, bronchial or tracheal lavage* must be treated with the following procedure:
  - Collect sputum into 50 mL single-use PP tubes with a screw cap.
  - In a biological safety cabinet, homogenize samples after mixing with equal volume of 4% NaOH solution. (*N-acetyl-L-cysteine may be added if required in the amount of 50-70 mg per sample).* Mix intensely with a tube rotator for 5-20 minutes (depending on the density of a sample).
  - Centrifuge samples at 3000 rpm (2800-3000 g) for 15 min and carefully discard the supernatant leaving 500-1000 µl in the tube. Resuspend sediment and transfer it into a 1.5 ml tube.
  - Centrifuge samples at 12000 rpm for 5-10 min, discard the supernatant and use the same 1,5 ml sample tube for DNA isolation from sample sediment.
- *tissue* (~1,0 gr) homogenized with mechanical homogenizer or scalpel, glass sticks, teflon pestles and dissolved in 1,0 ml of saline water or PBS sterile (1 volume of tissue to 1 volumes of saline solution). Vortex vigorously and incubate 30 min at room temperature. Transfer the supernatant into a new 1,5 ml tube;
- *whole blood* collected in either ACD or EDTA tubes;
- *liquor* stored in “Eppendorf” tube;
- *feces:* prepare 20% feces suspension by adding in 5 ml tube of 4ml of Saline Solution and 1,0 gr (approx. 1,0 ml) of feces. Vortex to get the homogeneous suspension and centrifuge for 5 min to 7000-12000g and using a micropipette with a plugged aerosol barrier tip transfer in a new sterile 1,5 ml tube 100 µl of the supernatant
- *sinovial liquid* stored in “Eppendorf” tube;
- *urine sediment* (use the intermedium part of stream);
- *prostatic liquid* stored in “Eppendorf” tube;
- *pleuric versament* stored in “Eppendorf” tube;
- *mycobacterium liquid culture* conserved in Trilon-B;

Specimens can be stored at +2-8°C for no longer than 48 hours, or freeze at -20°C to -80°C.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

The following kits are recommended:

- ⇨ **DNA-Sorb-B** (Sacace, REF K-1-1/B)
- ⇨ **DNA/RNA Prep** (Sacace, REF K-2-9)

Please carry out DNA extraction according to the manufacture's instruction. Add 10 µl of Internal Control (MTB IC) during DNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60–65°C until disappearance of ice crystals. Prepare required quantity of 1.5 ml polypropylene tubes including one tube for **Negative Control of Extraction**.
2. Add to each tube **10 µl** of **MTB IC** (**Internal Control)** and **300 µl** of **Lysis Solution**.
3. Add **100 µl** of **Samples** to the appropriate tube.
4. Prepare Controls as follows: add **100 µl** of **C–** (**Neg Control** provided with the amplification kit) to the tube labeled C*neg*.
5. Vortex the tubes and incubate for 5 min at 65°C. Centrifuge for 5-7 sec. If the sample is not completely dissolved it is recommended to re-centrifuge the tube for 5 min at a maximum speed (12000-16000 g.) and transfer the supernatant into a new tube for DNA extraction.
6. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 3 min at room temperature. Repeat this step.
8. Centrifuge all tubes for 30 sec at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
9. Add **300 µl** of **Washing Solution 1** to each tube. Vortex very vigorously and centrifuge for 30 sec at 5000g. Remove and discard supernatant from each tube.
10. Add **500 µl** of **Washing Solution 2** to each tube. Vortex vigorously and centrifuge for 30 sec at 10000g. Remove and discard supernatant from each tube.
11. Repeat step 10 and incubate all tubes with open cap for 5-10 min at 65°C.
12. Resuspend the pellet in **50 µl** of **DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
13. Centrifuge the tubes for 1 min at maximum speed (12000-16000 g). The supernatant contains DNA ready for amplification. If amplification is not performed in the same day of extraction, the processed samples can be stored at 2-8°C for at maximum period of 5 days or frozen at –20°/-80°C.

REAL TIME PCR PROTOCOL:

1. Prepare required quantity of reaction tubes (or PCR plate) for samples and controls.
2. Prepare in the new sterile tube for each sample **10*(N+1) µl** of **PCR-mix-1**, **5*(N+1) µl** of **PCR Buffer Flu** and **0,5*(N+1) µl** of **TaqF DNA Polymerase.** Vortex and centrifuge briefly.
3. Add to each tube **15 µl** of **Reaction Mix**.
4. Add **10 µl** of **extracted DNA** to appropriate tube.
5. Prepare for each panel 2 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of **MTB Diff C+** to the tube labeled Amplification Positive Control;
6. Insert the tubes in the thermalcycler.

Amplification

1. Create a temperature profile on your instrument as follows:

Step	Real Time PCR instruments[1,2]		
	Temperature, °C	Time	Cycles
Hold	95	15 min	1
Cycling	95	30 s	45
	65	30 s (detection)	
	72	30 s	

[1] *SaCycler-96™ (Sacace), Rotor-Gene™ 6000/Q (Corbett Research, Qiagen), iQ5™ (BioRad); Mx3005P™ (Stratagene), ABI® 7500 Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid)*
[2] *EcoqPCR® (Illumina)*

Fluorescence is detected at 65 °C in FAM/Green, JOE/Yellow/HEX/Cy3, ROX/Orange/Texas Red, and Cy5/Red fluorescence channels.

RESULTS ANALYSIS

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.

M. tuberculosis is detected on the Fam (Green) channel, *M.bovis* and *M. bovis BCG* on the Joe (Yellow)/HEX/TET/Cy3 channel, *M. bovis BCG* on the Rox (Orange)/TexasRed channel and IC on the Cy5 (Red) channel.

Results are accepted as relevant if positive and negative controls of amplification and extraction are passed (see table 1).

Table 1. Results for controls

Control	Stage for control	Fam (Green)	Joe (Yellow)/ HEX/Cy3	Rox/(Orange)/ TexasRed	Cy5 (Red)	Interpretation
NCE	DNA isolation	NEG	NEG	NEG	POS	Valid result
NCA	Amplification	NEG	NEG	NEG	NEG	Valid result
MTB Diff C+	Amplification	POS	POS	POS	NEG	Valid result

The following results are possible:

Fam (Green)	Joe (Yellow) HEX/Cy3	Rox/(Orange)/ TexasRed	Cy5 (Red)	Interpretation
+	+	+	+ /-	Mix M. tuberculosis + M. bovis BCG (+ M. bovis)
+	+	-	+ /-	Mix M. tuberculosis + M. bovis
+	-	+	+ /-	Mix M. tuberculosis + M. bovis BCG
+	-	-	+ /-	M. tuberculosis
-	+	+	+ /-	M. bovis BCG (+ M. bovis)
-	-	+	+ /-	M. bovis BCG
-	+	-	+ /-	M. bovis
-	-	-	+	Negative
-	-	-	-	Non valid

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

TROUBLESHOOTING

1. Weak or no signal of the IC (Cy5 (Red) channel) for the Negative Control of extraction.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow to the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting of the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - Improper DNA extraction.
        - ⇒ Repeat analysis starting from the DNA extraction stage
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and select for the IC detection the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the DNA extraction procedure.
2. Weak or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
3. Fam (Green), Joe (Yellow)/HEX/TET/Cy3 and Rox (Orange)/TexasRed signal with Negative Control of extraction.
    - Contamination during DNA extraction procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips between tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative Control of PCR (DNA-buffer).
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive control at last.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
	Expiration Date	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
IC	Internal Control	**RUO**	For Research Use Only

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* iQ5™ is a registered trademark of Bio-Rad Laboratories
* Rotor-Gene™ Technology is a registered trademark of Qiagen
* MX3005P® is a registered trademark of Stratagene
* ABI® is a registered trademark of Applied Biosystems
* SmartCycler® is a registered trademark of Cepheid
* EcoqPCR® is a registered trademark of Illumina

Sacace Biotechnologies Srl
via Scalabrini, 44 – 22100 – Como – Italy Tel +390314892927 Fax +390314892926
mail: info@sacace.com web: www.sacace.com